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UPTAKE OF NATURALLY OCCURRING PRIMARY AMINES BY MARINE ANNELIDS

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Rapid, carrier-mediated influx of small organic molecules across the body wall of marine invertebrates has been studied by a number of investigators. Recent literature is reviewed by Stephens (1972) who interprets available evidence as supporting a net influx which is large enough in many instances to represent a significant supplement to the nutrition of the animals concerned. This interpretation has been questioned by Johannes, Coward and Webb (1969). These investigators studied exchanges of amino acids between the flatworm, *Bdelloura,* and the environment and found a net efflux. Stephens and Schinske (1961) studied net exchanges of amino acids in a wide range of invertebrate material and reported a net influx. These results can be reconciled by noting the very different ambient concentrations used by the two sets of investigators and the relative insensitivity of efflux to changes in ambient concentration (as argued in Stephens, 1972). However, this analysis is somewhat indirect since it is based on separate measurements of influx and efflux using labelled substrates. The present contribution provides direct evidence for a rapid net influx of naturally occurring organic material and amino acids in two genera of the annelid infauna.

A fundamental gap in the literature concerning transepidermal uptake is the failure to establish any convincing link between this process and a quantitatively tenable source of available organic compounds in the environment. Speaking more generally, transepidermal uptake needs to be integrated into the general trophic structure of marine communities in order to aspire to a status other than that of an interesting curiosity of unknown significance. The present contribution also reports stimulation of production of available organic compounds in sediments as a result of the presence and activity of the annelid infauna. This represents the required linkage between uptake and production and is an initial step in the desired integration of the process into the overall trophic structure of the community.

This work was greatly faciliated by the introduction of a new reagent, fluorescamine, which allows rapid and sensitive measurement of primary amines in aqueous solutions. Fluorescamine reacts with primary amines, including amino acids and peptides, to form highly fluorescent derivatives (Udenfriend, Stein, Böhlen, Dairman, Leimgruber and Weigele, 1972; Weigele, DeBernardo, Tengi and Leimgruber, 1972). North (1975) describes the use of fluorescamine for measurement of naturally occurring primary amines in sea water. The simplicity of the procedure facilitates data collection, and the sensitivity of the reagent is sufficient to permit investigation of naturally occurring primary amines at concentrations as low as 2×10^{-7} moles per liter (expressed as equivalent glycine concentration).

Materials and Methods

Specimens of *Capitella capitata* and *Nereis diversicolor* were collected at Kysing Fjord, an estuary on the east coast of Jutland. The locality is described in detail in Muus (1967). Water temperatures decreased from 16° to 7° C during the period of study. Animals were maintained either in an aquarium room at 15° C or a refrigerator at 6° C. Specimens for use in studies of uptake of primary amines were held in small vessels containing sea water or in aquaria containing sediment collected from Kysing Fjord.

Sediment cores were obtained from Kysing Fjord and from other areas for comparison. A clear plastic cylinder, 25 mm internal diameter and 15 to 20 cm in length, was forced into the sediment, corked and carefully removed to obtain samples for study with a minimum of disturbance of existing stratification. Interstitial water from sediment and sediment cores was expressed under 2 to 3 atmospheres of N_2 using a pressure filtration apparatus and Millipore filters of 0.45 micron pore size. This procedure expressed 60 to 70 percent of the water contained in the sediments studied.

Amino acids and naturally occurring primary amines were measured as follows. A sample of 0.5 ml was buffered to pH 9.25 with 0.5 ml of 0.5 N borate buffer. 0.5 ml of 200 mg fluorescamine per liter of acetone was added to the buffered sample mixing vigorously. The resulting precipitate was redissolved by adding 1.5 ml glass distilled water. Fluorescence was measured at 480 nm using an excitation wavelength of 390 nm. Glassware was acid washed and rinsed with glass distilled water. Fluorescamine positive material is present in Millipore filters; this was eliminated by soaking in dilute acid and in glass distilled water. High concentrations of sulfide ion interfere with the analysis. Selected samples from sediment core interstitial water were analyzed with and without addition of $CdCl_2$ to precipitate sulfide to verify that natural levels of sulfide did not interfere. Sea water solutions of amino acids were prepared using natural sea water passed through a Millipore filter. Natural primary amines were less than 5×10^{-7} moles per liter expressed as glycine equivalents.

Redox potentials were measured using a polished platinum wire electrode. The electrode was cleaned before each series of measurements and standardized using quinhydrone at pH 6.5. Water content of sediment was determined by weighing after drying at 105° C. Organic content was determined after ignition at 470° C. Free amino acids in samples of interstitial water were desalted and chromatographed using the TLC procedure described by Clark (1968). The samples were lyophilized and chromatographed in California.

Results

Concentration of primary amines in interstitial water

Concentration is expressed as glycine equivalents. As noted by North (1975) specific fluoresence of fluorescamine derivatives of amino acids differs. Using the procedure described above, I investigated the fluorescence of glycine, serine, glutamate and aspartate solutions in natural sea water. Fluorescence of the fluorescamine derivatives was found to be 1 : 0.87 : 0.67 : 0.47 for these amino acids at a

pH of 9.25. Other primary amines (*e.g.*, peptides) have a rather lower fluorescence at this pH and ammonia does not give any fluorescence. North reports that the specific fluorescence of lysine, arginine and ornithine is slightly higher than that of glycine. However, these amino acids are not major constituents of primary amines in interstitial water. Thus the use of glycine equivalents represents a conservative estimate of molar concentrations of naturally occurring primary amines.

Primary amines were measured in a total of 32 samples of interstitial water from the top three centimeters of sediment. Concentrations ranged from a minimum of 29 to a maximum of 103 micromoles per liter expressed as glycine equivalents. The average value was 50 micromoles per liter. Deeper levels of the sediment cores exhibited lower concentrations of primary amines. Thus the concentrations measured at depths between 6 and 9 centimeters ($n = 8$) ranged from 16 to 40 micromolar with an average of 23. Duplicate measurements on the same extract were excellent, normally agreeing within 2 percent as did duplicates obtained by extracting split cores. However, multiple sampling over an area of a square meter indicated a patchy distribution with individual determinations of primary amines from different cores differing by as much as 50 percent. There was no obvious macroscopic correlate of this patchiness.

Because of the variation in level of primary amines in the cores examined, values for surface samples overlap with values at deeper levels, as noted above. Table I expresses data from measurements of primary amines in successive layers of natural cores collected from Kysing Fjord. The table gives the ratio of primary amines in the 3 to 6 cm layer and the 6 to 9 cm layer to primary amines in the surface 3 cm. The decrease in primary amine concentration with depth is a consistent feature of these measurements.

Redox potentials of each core were measured at 5 mm intervals from the sur-

TABLE I

Ratios of the concentration of primary amines in interstitial water of sediment cores at the indicated depth to concentration of primary amines in the interstitial water of the surface 3 cm of the cores.

	Depth (cm)	Primary amines (micromols/l)	Ratio of amine concentrations	n
Natural cores	0–3	51.0 ± 24.8		11
	3–6	27.4 ± 14.9	0.57 ± 0.18	11
	6–9	23.1 ± 9.8	0.50 ± 0.14	8
Sieved cores	0–3	57.6 ± 28.8		6
	3–6	34.8 ± 15.5	0.61 ± 0.11	6
	6–9	26.1 ± 9.3	0.50 ± 0.12	6
Cores plus *N. diversicolor*	0–3	43.9 ± 26.0		6
	3–6	38.6 ± 24.2	0.90 ± 0.17	6
	6–9	39.9 ± 17.9	0.93 ± 0.37	6
Irrigated cores	0–3	55.4 ± 18.6		6
	3–6	51.6 ± 14.2	0.96 ± 0.18	6
	6–9	55.0 ± 21.1	1.00 ± 0.24	6

face. All cores examined were quite reduced, achiving an E_h of 0 mv from 3 to 12 mm below the surface and an E_h of -140 to -250 mv at a depth of 3 cm.

Net influx of naturally occurring amines and of amino acids

Single individuals of *N. diversicolor* and groups of 10 to 15 *C. capitata* were exposed to samples of interstitial water or to solutions of glycine, serine, glutamate and aspartate. Specimens of *Nereis* ranged in weight from 71 to 237 mg and the groups of *Capitella* were selected to weigh approximately 100 mg collectively.

Primary amines were determined initially and at suitable intervals after immersing the worm or worms in a sample. The general result was an exponential decrease in concentration of primary amines with time. Figure 1 is a semilog plot of the concentration of primary amines in interstitial water from a surface (0 to 3 cm) sample taken from Kysing Fjord and exposed to a group of *C. capitata*. Weight of worms was 111 mg and the volume of the water sample was 10 ml. In this particular case, the worms had been selected and exposed previously to six aliquots of the same interstitial water sample at approximately six-hour intervals over the preceding two days. Sufficient streptomycin sulfate and penicillin was added to give concentrations of 100 mg/liter and 10^6 IU/liter respectively. The rate of influx expressed as micromoles of glycine equivalents per gram per hour can be estimated from the slope of the semilog plot and is approximately 0.81 from a concentration of 50 micromoles (glycine equivalent). This is not significantly different from the rate of influx observed when naive worms are exposed for the first time to the same interstitial water sample without antibiotics (see Table II).

As indicated in Table II naturally occurring amines were also available to *Nereis* at a comparable rate. However, the data for the two species differ. The exponential decline in concentration of primary amines can be followed down to

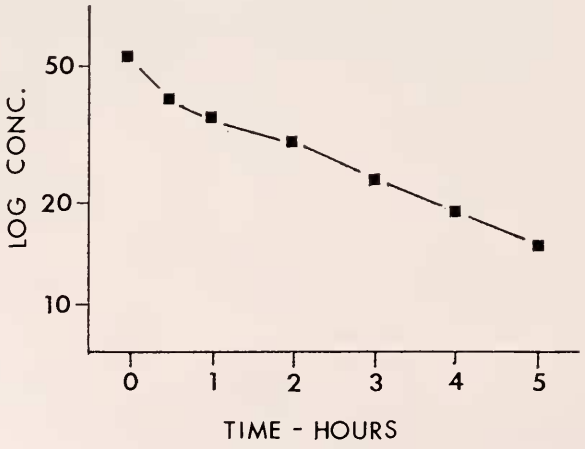

FIGURE 1. Disappearance of naturally occurring primary amines in the presence of *Capitella capitata*. Initial concentration is 54 micromoles per liter (glycine equivalents); final concentration is 15 micromoles per liter. See text for details.

TABLE II

Net influx of primary amines from interstitial water and net influx of amino acids. Rates are expressed as micromoles per hour per gram worm from an ambient concentration of 50 micromoles per liter.

Capitella capitata		*Nereis diversicolor*	
amines*	0.89	amines*	0.75
amines**	0.81	glycine	0.84
glycine	0.75	serine	0.99
serine	0.85	glutamate	0.05
glutamate	0.82	aspartate	0.03
aspartate	0.85		

* Expressed as micromoles glycine.
** Incubated for 48 hours with streptomycin and penicillin, seventh exposure to amines.

concentrations of 2 to 3 micromoles per liter in the case of *Capitella*. At this point influx ceases and concentrations may increase to 4 to 5 micromoles over the course of several hours. In contrast, external concentration of amines decreases exponentially in the presence of *Nereis* until a value of 30 to 40 percent of the initial concentration has been reached. The rate of disappearance declines slowly. This is to some extent independent of initial concentration since the same result is obtained if the naturally occurring amines are diluted to present an initial concentration of 10 micromoles.

Net influx of known acids was examined using *Nereis* and *Capitella*. Initial concentrations were 50 micromoles per liter (10 in some experiments). Table II gives rates of net influx from a concentration of 50 micromoles per liter. *Capitella* obtained glutamate and aspartate from solution at rates comparable to those observed for glycine and serine. The values of glutamate and aspartate uptake are on an order of magnitude lower for *Nereis*. It is not clear that *Nereis* could take up these compounds at all. The data show a consistent downward trend but the experiments were relatively long (8 hours) and no antibiotics were used. Thus *Nereis* makes little or no use of the free dicarboxylic amino acids present in the interstitial water while *Capitella* can apparently do so. *Nereis* reduces the ambient concentration of glycine or serine to minimum levels of 1 to 3 micromoles per liter; *Capitella* does so for all of the four amino acids employed.

Free amino acids in interstitial water samples

Fifteen amino acids were identified from two samples of interstitial water which were desalted, chromatographed and eluted for quantitative estimates. The order of concentration in the two samples was respectively ala, glu, gly, ser, val, asp for the first and ala, glu, asp, gly, ser, val for the second. Amino acids accounted for virtually all of the ninhydrin positive material in the desalted samples. The data do not permit conclusions about the extent to which fluorescamine and ninhydrin determinations of primary amines and of amino acids agree. An aliquot of one of these samples which had been exposed to a group of *Capitella* for five hours was also chromatographed. Free amino acids were markedly lower in concentration. The amino acids ala, gly, glu, and ser were present in that order by visual estimate plus small amounts of lys, arg, and his.

Artificial sediment cores

Eighteen artificial sediment cores were prepared by filling clear plastic tubes, 25 mm in internal diameter, to a depth of about 15 cm with sediment freshly collected at Kysing Fjord. The sediment was passed through a 1 mm mesh seive to remove macrofauna and large particles. The sediment contained 0.86% organic matter by weight as determined by ignition at 470° C. After preparation, the artificial cores were kept submerged in aerated sea water in an aquarium at 15° C. The bottom of each tube was firmly corked with a rubber stopper; the top was exposed to the aerated sea water.

All of the artificial cores became oxidized at the surface and assumed a redox profile comparable in detail to that described earlier for natural cores in this study. The first E_h measurements were taken four days after constructing the cores.

Six cores were left undisturbed as described. A single specimen of *N. diversicolor* (weight range from 96 to 243 mg) was added to each of six cores immediately after preparation. A piece of 1 mm mesh nylon screen was tied over the mouth of each tube to retain the animals. The remaining six cores were exposed to a subsurface flow of aerated sea water at a rate of 12 to 15 ml per hour. The sea water was siphoned from an aerated tank and entered the core through a Pasteur pipet. Flow rate was controlled by a tubing clamp. The tip of the pipet was inserted 6 to 7 cm below the surface of the core. The pipet was surrounded by a sleeve of nylon screen extending to the surfac of the core to minimize mechanical disturbance of the sediment as a result of the water flow.

The cores containing *Nereis* and those artificially irrigated with aerated sea water developed much the same redox profiles as the natural cores and the undisturbed artificial cores with minor differences to be described. Figure 2 shows the redox profile of a core containing a specimen of *Nereis*. In this case, as in all of the worm-containing cores, a layer of bright yellow sediment formed immediately around the burrow constructed by the worm. The solid line shows redox potential measurements away from the immediate area of the burrow; the dotted line shows measurements obtained when the platinum electrode was thrust down the burrow itself. Figure 3 shows a redox profile obtained from a core irrigated with aerated sea water. The two open squares are measurements of E_h from the same core taken with a freshly cleaned platinum electrode immediately after exposing bright yellow sediment to the air at the depths indicated.

The redox profiles of natural cores and those assumed by artificial cores imply transition from aerobic to anaerobic conditions within the top 3 cm of the sediment (Fenchel, 1971). The platinum electrode used for measurement of redox potentials is easily poisoned. However, measurements along worm burrows or in the freshly exposed, bright yellow regions of subsurface sediment in irrigated cores though higher than the surrounding sediment were still strongly negative. This suggests that oxygen introduced into deeper layers of the sediment, artificially or as a result of the activity of the worms, is transient and results in oxidation of sulfide in the immediate vicinity. In effect, an additional aerobic-anerobic transition area is produced.

Table I presents the concentration of primary amines at various depths and lists ratios of the primary amines found at depths of 3 to 6 cm and 6 to 9 cm to primary amines in the surface 3 cm for the three types of artificial cores. The

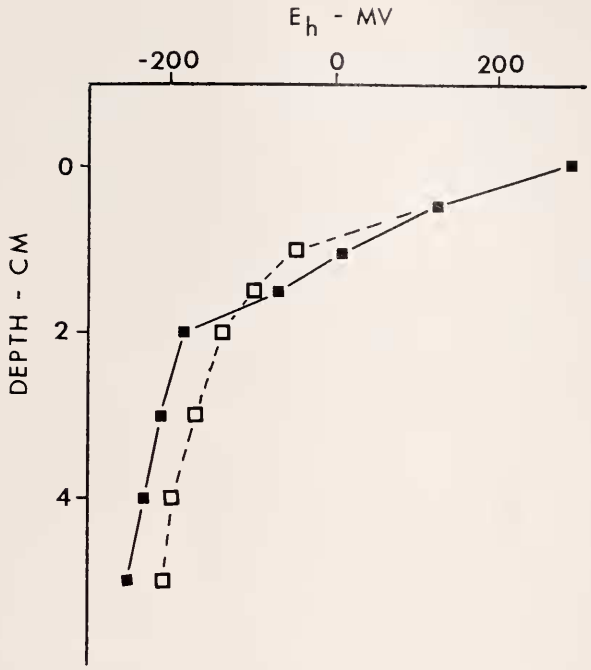

FIGURE 2. Redox profile of an artificial sediment core containing a specimen of *Nereis diversicolor*. The solid line connects measurements of redox potential of the sediment. The dotted line connects measurements of redox potential along the burrow of the worm.

content of primary amines did not decrease with depth in cores containing worms or in cores receiving a flow of aerated sea water. The average content of primary amines in the surface 3 cm of the different types of artificial cores did not differ significantly. Undisturbed and "aerated" cores averaged 57.6 and 55.4 micromoles per liter respectively. Cores containing worms averaged 43.9 micromoles per liter (range 23.3 to 92.5). Thus the increased ratios in Table I result from an increase in primary amines in the deeper layers of the artificial cores containing worms or receiving a subsurface supply of aerated sea water.

DISCUSSION

There is a net flux of naturally occurring primary amines into both *Capitella capitata* and *Nereis diversicolor*. The flux is almost certainly transepidermal though the present work does not directly confirm this deduction. If intake across the gut surface were to be postulated, the measured rates of disappearance of primary amines would imply passage of at least fifteen to twenty times the volume of the worms through their digestive tract each hour. Earlier demonstration for other annelids of rapid transepidermal influx using ^{14}C-labelled compounds at rates unimpaired by ligation of mouth and anus (*e.g.*, Stephens, 1963) as well as examination of influx in isolated epidermal preparations (*e.g.*, Chien, Stephens and Healy, 1972) support this interpretation of the present observations.

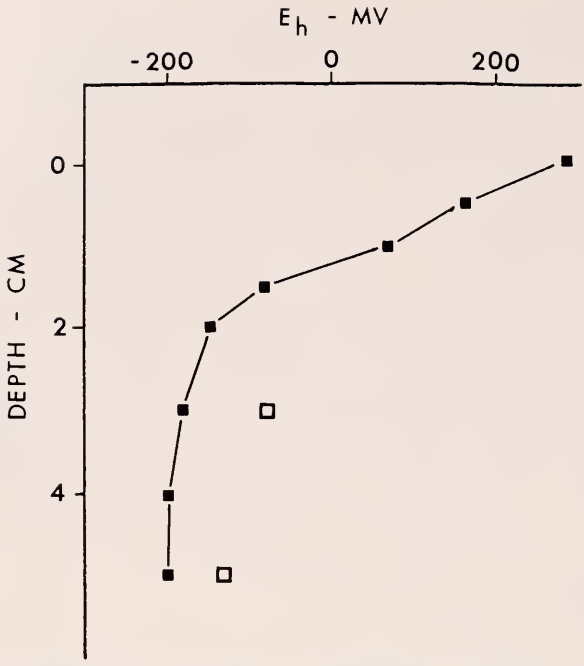

FIGURE 3. Redox profile of an artificial sediment core irrigated with aerated sea water. The solid line connects measurements of redox potential of the sediment. Open squares are measurements of the redox potential of freshly exposed, bright yellow sediment at the indicated depths.

The data strongly suggest that the worms are the agents for disappearance of the primary amines rather than contaminating microflora. This is supported by the fact that antibiotics failed to modify rates of net flux (Figure 1 and Table II). It is also supported by the fact that glutamate and aspartate were removed rapidly from solution by *Capitella* and slowly or not at all by *Nereis*. No antibiotics were present in the solutions in these experiments. It is unlikely that contaminant microorganisms were present and active in the first case and absent or inactive in the second, since the two organisms were collected from the same locality and the same sediment samples and were treated similarly.

The entry of naturally occurring primary amines occurs at rates which are of the same magnitude as the total requirement of the animals for reduced carbon. As noted above, use of equivalent glycine concentration units is a conservative estimate of abundance of these compounds. Assuming that *Capitella* is taking in the six most abundant amino acids found in interstitial water (average molecular weight, 111) the net influx reported in Table II represents 90 to 99 micrograms per gm worm per hour. Inclusion of a correction for differences in specific fluorescence would increase this figure. The same calculation for *Nereis* produces a lower figure since glutamate and aspartate are not utilized (average molecular weight, 96; hourly influx, 72 micrograms per gm). The conversion factor for a mixture of amino acids to oxygen consumption is one microgram equivalent to one

microliter. Oxygen consumption data for marine infaunal annelids tabulated in standard handbooks covers a range of about 8 to 135 microliters O_2 per gm per hour.

This discussion is subject to the caveat that primary amines are not necessarily free amino acids. Previous work with annelids and other groups of soft-bodied invertebrates has shown that amino acids entering via the transepidermal route enter all aspects of the metabolism of the organism involved (reviewed in Stephens, 1972). It is possible that the naturally occurring primary amines include compounds that can enter the worms but cannot participate in their metabolism. The data from chromatography are of some help but are not conclusive in resolving this issue. Thus, little or no ninhydrin-positive material remained at the origin in chromatograms of desalted samples of interstitial water. All ninhydrin-positive spots were identifiable as amino acids. This suggests free amino acids make up the greatest part of the naturally occurring primary amines. This suggestion is also supported by the agreement between the present report of levels of primary amines and previous reports of free amino acid concentrations from interstitial water samples (Stephens, 1963; Stephens, 1972). Ammonia does not react with fluorescamine. Thus the present interpretation of primary amines as amino acids is not likely to be an overestimate of rates of entry of reduced carbon. However, it is possible that some amines may be present which are not available to the worms metabolically.

The potential contribution of this process to the nutrition of *Capitella* is of particular interest. There is mounting evidence for the view that the only significant primary decomposers of plant material in the sea are microbial (reviewed in Fenchel, 1972). Thus "detritus-feeding" metazoans are dependent on microbial activity; a number of such animals have been shown to utilize the microbial flora as a food source rather than detritus itself. Conversely, there is no convincing demonstration of the ability of a "detritus-feeder" to digest detritus. However, there are cases, one of which is *Capitella*, of animals where careful examination has failed to produce clear evidence of ability to utilize either detritus or the microbial flora. In aquaria experiments *Capitella* selectively feeds on decaying fragments of *Ulva* in the reduced sediment. However, direct observation under the microscope of stained fragments shows that many bacteria pass undigested (or they are added from the gut flora). The fragments themselves are also relatively little digested. In agreement, the total organic matter as well as the content of protein of the fragments are only slightly lower than values obtained with control fragments picked from the sediment (personal communication, J. Hylleberg, University of Aarhus). Though the possibility that *Capitella* is obtaining some sustenance from ingestion of sediment is not totally excluded, it is attractive to visualize the intake of dissolved material as a large component of its nutritional strategy. These comments do not apply to *Nereis*. Worms in this genus have been shown to digest microbial flora, diatoms and algal tissue, and the worms are opportunist carnivores and occasional filter feeders. However, the present work indicates that *N. diversicolor* is also capable of obtaining supplementary nutrition from dissolved compounds in the interstitial water.

Generation of primary amines in cores containing *Nereis* or irrigated with sea water is of great interest. The following interpretation of this observation is suggested. The redox potentials of natural cores and undisturbed artificial cores

indicate that primary amines are most concentrated in the region which includes the transition zone from aerobic to anaerobic conditions. The presence of a worm or artificial irrigation has the effect of increasing the surface area of this transition zone by folding and extending this zone into the deeper layers of the sediment. Both *Capitella* and *Nereis* are known to irrigate their burrows (Lindroth, 1941). The burrowing activity of the infauna and their behavior in irrigating their burrows serves both to supply them with oxygen for their metabolism and to generate primary amines which are then available as a nutritional source. Thus the ability to obtain and utilize primary amines from a solution is coupled to the generation of these compounds.

The observations reported here do not permit identification of the source of the additional primary amines. However, the presence of detritus-feeding invertebrates has been shown to promote microbial activity in marine sediments. This is true whether one measures oxygen consumption of the microflora (Hargrave, 1970) or counts the number of bacteria per unit surface of detritus particles (Fenchel, 1972). It is likely that the present observation of an increase in primary amines is also related to stimulation of microbial activity. Jørgensen and Fenchel (1974) have shown that the activity of bacteria which reduce sulfate to sulfide and reoxidize the resulting sulfide dominates the metabolism of reduced marine sediments. Furthermore, this activity is most intense in the transition layer between aerobic and anerobic conditions. Thus it is attractive to speculate that the increase in area of this transition zone resulting from the irrigation by the worms, the intense microbial activity, and the increase in primary amines are related phenomena.

This work was carried out during tenure as a Senior Science Fellow of NATO in the autumn of 1974. I wish to express my sincere thanks to Professor Tom Fenchel for use of laboratory facilities at the University of Aarhus and for helpful discussions, to Dr. Jørgen Hylleberg for his interest and help and for permission to allude to as yet unpublished observations of feeding in *Capitella*, and to Dr. Bo Barker Jørgensen for help and advice and for obtaining sediment cores from the Limfjord. I also wish to acknowledge with thanks the contribution of Patricia Wheeler who chromatographed the amino acids in interstital water samples.

Summary

1. The interstitial water of inshore sediments on the Danish coast contains rather high concentrations of primary amines. The average concentration observed was 50 micromoles per liter expressed as equivalent glycine concentration.

2. The concentration of primary amines decreased in deeper layers of the sediment.

3. Naturally occurring primary amines from interstitial water are taken up rapidly by *Nereis diversicolor* and *Capitella capitata*. The rate of net influx is of the same order of magnitude as the total requirement for reduced carbon provided primary amines are interpreted as principally consisting of free amino acids.

4. Net influx of known amino acids was also examined. *Capitella* takes up glycine, serine, glutamate and aspartate at rates comparable to those observed for naturally occurring amines in the interstitial water. *Nereis* takes up glycine

and serine at comparable rates but obtains glutamate and aspartate from solution very slowly if at all.

5. Subsurface irrigation of sediment samples with aerated sea water increases the concentration of primary amines in the interstital water of deeper sediment layers. The same effect results when *Nereis* constructs and irrigates a burrow.

6. These observations support the hypothesis that a large net transepidermal influx of small organic compounds occurs in members of the annelid infauna. Furthermore, this ability to obtain and utilize primary amines from solution is associated with behavior which results in generating additonal primary amines.

7. It is suggested that the construction of burrows and their irrigation by the infauna serves to increase the surface area of the transition zone between aerobic and anaerobic conditions by extending it into deeper sediment layers. The observed increase in primary amines may be related to the intense microbial activity which characterizes this transition zone.

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